



# Modified combined disc test (mCDT): a novel, labor-saving and 4 times cheaper method to differentiate Class A, B and D carbapenemase-producing *Klebsiella* species

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## ABSTRACT

Carbapenemase-producing organisms have been an immense public health problem in recent years. Combined disc test (CDT) is a simple and widely used phenotypic method for carbapenemase detection, especially in developing countries. This study evaluates the performance of modified combined disc test (mCDT), a novel and 4 times cheaper method than CDT. In total, 572 (15.5%) *Klebsiella* spp. including 81 (14.2%) carbapenemase producers were isolated from 3993 clinical samples. Both mCDT and CDT showed similar sensitivity, specificity, positive predictive value, and negative predictive value for the differentiation of Class A, B, and D carbapenemase-producing *Klebsiella* spp.

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## 1. Introduction

The enzyme carbapenemase renders bacteria resistant to nearly all routine antibiotics and is a very important cause of treatment failure, increased hospital stay, and high mortality rate (Casteneda and Luengas, 2018; Kaur et al., 2015). Carbapenemase-producing *Klebsiella* spp. have been documented around the globe and are associated with high mortality rate to be 57.6% (Pagano and Cairra, 2014) and 68% (Veeraraghavan and Shankar, 2017). Thus, timely and accurate detection of carbapenemase-producing bacteria is of utmost importance for better treatment modality and to reduce mortality rate (Djahmi et al., 2014). Carbapenemase can be classified as Ambler Class A (encoded by KPC gene), Class B (encoded by NDM, IMP, and VIM genes), and Class D (encoded by OXA-48 like gene). Class A and D enzymes have serine-based hydrolytic activity (hence called serine carbapenemase), whereas Class B needs metal, e.g., zinc, for its activity (thus known as metallo-beta-lactamase; MBL) (Aqel et al., 2017; Kumar et al., 2018a). Combined disc test (CDT), being cheaper than genotypic methods, is the most widely used test for carbapenemase detection in low- and mid-income countries (Hrabak et al., 2014). This study was conducted with the aim to develop and evaluate a 4 times cheaper modification

of CDT for the detection and differentiation of Class A, B, and D carbapenemase-producing *Klebsiella pneumoniae* and *Klebsiella oxytoca*, devoid of compromising with sensitivity and specificity.

## 2. Materials and methods

### 2.1. Bacterial isolation

A total of 3693 nonduplicate clinical samples (sputum, urine, pus, blood, high vaginal swab) were processed for the isolation of *Klebsiella* species as per standard operating procedures (Koneman et al., 2005). Isolates were subjected for the detection of serine carbapenemases (Class A and D) and MBL (Class B) carbapenemase by screening method and further confirmation by combined disc test (CDT) and polymerase chain reaction (PCR). All genotypically confirmed positive strains and 100 non-carbapenemase-producing strains were finally subjected to mCDT.

### 2.2. Carbapenemase screening test

*Klebsiella* isolates, showing the zone diameter < 22 mm to meropenem (10 µg) disc by Kirby Bauer disc diffusion method as per CLSI guidelines, were considered suggestive of carbapenemase producers (CLSI, 2017).

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### 2.3. Confirmatory tests

#### 2.3.1. Combined disc test

Screen positive strain was inoculated in peptone water, incubated at 37 °C for 2–4 h, and adjusted to 0.5 McFarland Standard (equivalent to  $1.5 \times 10^8$  CFU/mL). This test suspension was inoculated as lawn culture on Mueller Hinton agar (MHA) plate. Then, 1 disc of temocillin (30 µg) and 4 discs of meropenem (10 µg) –1 disc without any inhibitor, a second disc with phenyl boronic acid (PBA) (400 µg), a third disc with EDTA (292 µg), and a fourth disc with both PBA plus EDTA—were placed, and plate was incubated at 37 °C for 18–24 h.

Test strain showing more than 5 mm zone diameter around meropenem with PBA and meropenem with both PBA and EDTA than zone diameter around meropenem disc alone was considered serine (KPC) carbapenemase producer.

Test strain showing more than 5 mm zone diameter around meropenem with EDTA and meropenem with both PBA and EDTA than zone diameter around meropenem disc alone was considered MBL producer.

Test strain showing no increase in zone diameter with inhibitors and < 10 mm zone diameter around temocillin was considered serine (OXA-48 like) carbapenemase producer (Dijk and Voets, 2014).

#### 2.3.2. Polymerase chain reaction

Screen positive strains were also subjected to conventional PCR method for the detection of Class A (KPC gene), Class B (VIM, IMP, and NDM-1 genes), and Class D (OXA-48 gene) using following primers (Farzin et al., 2017; Kumar et al., 2018b):

F-5'-TCGAACAGGACTTTGGCG-3' & R-5'-GGAACCAGCGCATTTTTGC-3' for KPC;  
 F-5'-GTTTGGTCGCATATCGCAAC-3' & R-5'-AATGCGCAGCACCAGG ATAG-3' for VIM;  
 F-5'-GAAGCGTTTATGTTTCATAC-3' & R-5'-GTAAGTTTCAAGAGTG ATGC-3' for IMP;  
 F-5'-GCATAAGTCGCAATCCCCG-3' & R-5'-CTTCCTATCTCGACAT GCCG-3' for NDM-1.  
 F-5'-GCGTGGTTAAGGATGAACAC-3' & R-5'-CATCAAGTTCAACCA ACCG-3' for OXA-48.

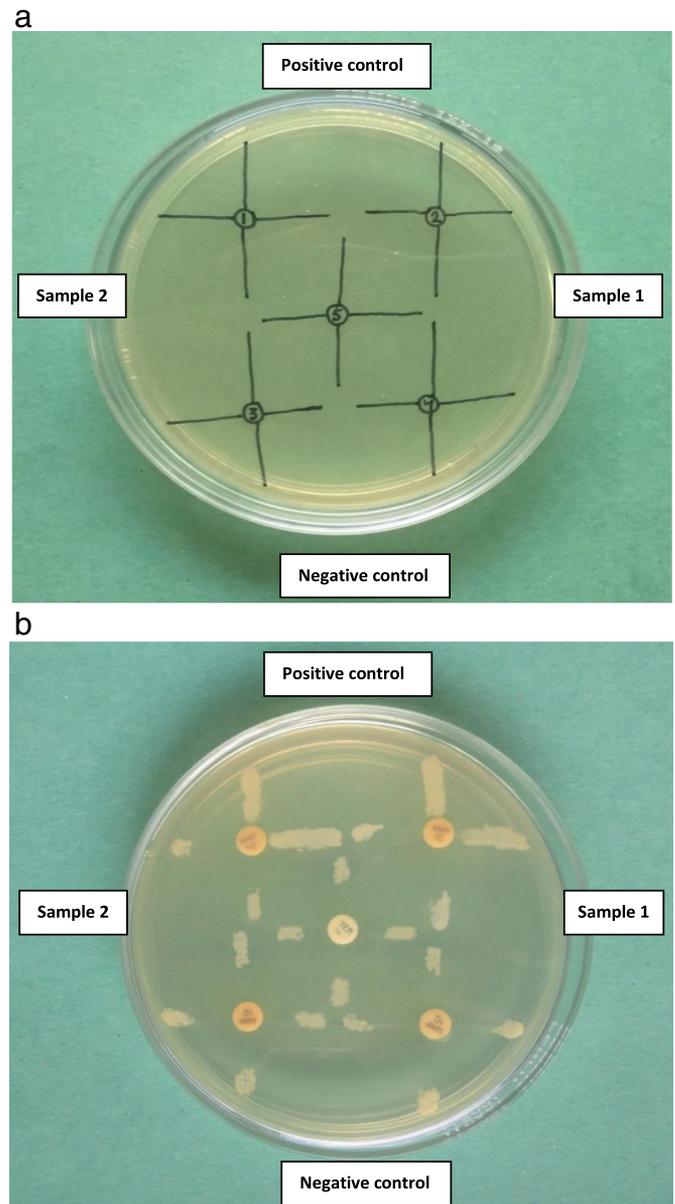
#### 2.3.3. Modified combined disc test (mCDT)

A MHA plate was taken, and 1 disc of temocillin (30 µg) and 4 discs of meropenem (10 µg)—1 disc without any inhibitor, a second disc with PBA (400 µg), a third disc with EDTA (292 µg), and a fourth disc with both PBA plus EDTA—were placed. A sterile cotton swab was dipped in the suspension (similar as in CDT), and then extra suspension is removed by pressing the swab against the inner wall of the tube. Then, the test strain was streaked in a straight line of around 14-mm length from the edge of all 5 discs in the same directions. Four strains were streaked at a time in 4 directions, and plate was incubated at 37 °C for 18–24 h (Fig. 1a, b).

Test strain showing more than 2.5 mm growth inhibition near meropenem with PBA and meropenem with both PBA and EDTA than growth inhibition near meropenem disc alone was considered serine (KPC) carbapenemase producer.

Test strain showing more than 2.5 mm growth inhibition near meropenem with EDTA and meropenem with both PBA and EDTA than growth inhibition near meropenem disc alone was considered MBL producer.

Test strain showing no increase growth inhibition with inhibitors and < 3 mm growth inhibition near temocillin was considered serine (OXA-48 like) carbapenemase producer (Table 1).



**Fig. 1. a.** Uninoculated MHA plate with streak marking for mCDT test (disc 1; meropenem alone, disc 2; meropenem plus PBA, disc 3; meropenem plus EDTA, disc 4; meropenem plus PBA plus EDTA, disc 5; temocillin). **b.** mCDT test is showing positive control (MBL), negative control, and 2 test strains around all 5 antibiotic discs. Sample 1 is showing < 8 mm growth inhibition to disc 1 and disc 2, growth inhibition near disc 3 and 4 is 2.5 mm more than disc 1, and growth near disc 5 is more than 3 mm; thus, sample 1 is MBL (Class B) producer. Sample 2 is showing growth inhibition > 8 mm in all the 5 discs; thus, sample 2 is noncarbapenemase producer.

### 2.4. Quality control

*Klebsiella pneumoniae* ATCC 2146 and *Klebsiella pneumoniae* ATCC 1705 were used as positive controls for MBL and serine carbapenemase detection, respectively.

## 3. Results

### 3.1. Sample processing and bacterial isolation

From 3693 clinical specimens, a total of 572 (15.5%) *Klebsiella* strains were obtained, of which 81 (14.2%) were carbapenemase producers including 67 (81.7%) *Klebsiella pneumoniae* and 14 (18.3%) *Klebsiella*

**Table 1**  
Interpretation of mCDT and CDT for the differentiation of Class A, B, and D carbapenemase-producing *Klebsiella* spp.

| Method | Carbapenemase classes | Total 5 discs [4 meropenem (10 µg) and 1 temocillin (30 µg)] |   |   |   |                     |
|--------|-----------------------|--|---|---|---|---------------------|
|        |                       | Meropenem alone (disc 1)                                     | Meropenem + PBA (disc 2)                  | Meropenem + EDTA (disc 3)                 | Meropenem + PBA + EDTA (disc 4)           | Temocillin (disc 5) |
| mCDT   | Class A               | <8 mm  | ≥2.5 mm more inhibition than disc 1       | No change                                 | ≥2.5 mm more inhibition than disc 1       | >3 mm               |
|        | Class B               | <8 mm  | No change                                 | ≥2.5 mm more inhibition than disc 1       | ≥2.5 mm more inhibition than disc 1       | >3 mm               |
|        | Class D               | <8 mm  | No change                                 | No change                                 | No change                                 | <3 mm               |
| CDT    | Class A               | <22 mm   | ≥5 mm more zone of inhibition than disc 1 | No change                                 | ≥5 mm more zone of inhibition than disc 1 | >10 mm              |
|        | Class B               | <22 mm   | No change                                 | ≥5 mm more zone of inhibition than disc 1 | ≥5 mm more zone of inhibition than disc 1 | >10 mm              |
|        | Class D               | <22 mm   | No change                                 | No change                                 | No change                                 | <10 mm              |

*oxytoca*. Furthermore, out of 81 carbapenemase-producing *Klebsiella* spp., 12 (14.8%) were Class A, 67 (82.7%) Class B, and 2 (2.5%) were Class D positive strains.

The majority of carbapenemase producing *Klebsiella* species (34; 42% of 81) were isolated from sputum samples followed by urine (22; 27.2% of 81) and pus samples (17; 21% of 81) (Table 2).

### 3.2. Comparison of CDT and mCDT

Modified combined disc test utilized 21 MHA plates (90 mm), 84 meropenem discs (with/without inhibitors), and 21 temocillin discs to process 84 (81 test strains, 2 positive controls, and 1 negative control) strains. In contrast, combined disc test required 84 MHA plates, 336 meropenem discs (with/without inhibitors), and 84 temocillin discs. However, both tests equally detected 76 (93.8%) carbapenemase-producing *Klebsiella* spp. (Table 3).

### 3.3. Performance of mCDT

Modified combined disc test, when compared with PCR results, showed overall sensitivity and specificity to be 93.8% (76/81) and 100%, respectively. Sensitivity of this modification to detect Class B

carbapenemase was 95.5% compared to 83.3% for Class A with 100% specificity (Table 4). Diagnostic performance of CDT also illustrated similar results as of mCDT when compared with PCR.

## 4. Discussion

This study evaluates a novel and cheaper modification of combined disc test for the detection of Class A, B, and D carbapenemase production.

In this study, 14.2% (81/572) of *Klebsiella* strains were carbapenemase producers. Similar results of 14.4% and 12% carbapenemase production in *Klebsiella* spp. have been published previously (Chakkarapani et al., 2014; Chauhan et al., 2015). We observed that 14.8%, 82.7%, and 2.5% carbapenemase producers were belonging to Class A, B, and D, respectively. Several studies conducted in India revealed 14.3% (Rachna et al., 2014) prevalence of Class A carbapenemase; 79.7%, 83.3%, and 94.6% (Varsha et al., 2013; Rachana et al. 2014; Naresh et al., 2015) of Class B; and 13% (Veeraraghavan and Shankar, 2017) of Class D carbapenemase in *Klebsiella* spp.

These finding may be associated with the fact that the Indian sub-continent is the focal point of NDM-1 (Class B) positive strains (Khan et al., 2017). A study by Laura et al. (2016) also reported that the most

**Table 2**  
Sample wise distribution of PCR positive Class A, B, and D carbapenemase-producing *Klebsiella* spp.

|   |                         | Specimen type n (%) |             |              |            |            |
|---|-------------------------|---------------------|-------------|--------------|------------|------------|
|   |                         | Sputum              | Pus         | Urine        | Blood      | HVS        |
| Total samples (n = 3693)                                      |                         | 977 (26.5%)         | 708 (19.2%) | 1521 (41.2%) | 268 (7.3%) | 219 (5.9%) |
| <i>Klebsiella</i> spp. isolated (n = 572)                     |                         | 219 (38.3%)         | 121 (21.2%) | 168 (29.4%)  | 42 (7.3%)  | 22 (3.8%)  |
| Total carbapenemase-producing <i>Klebsiella</i> spp. (n = 81) |                         | 34 (42%)            | 17 (21%)    | 22 (27.2%)   | 5 (6.2%)   | 3 (3.7%)   |
| Carbapenemase-producing <i>Klebsiella pneumoniae</i> (n = 67) | <b>Class A (n = 9)</b>  | 3 (33.3%)           | 2 (22.2%)   | 4 (44.4%)    | 0          | 0          |
|   | <b>Class B (n = 56)</b> | 24 (42.9%)          | 12 (21.4%)  | 14 (25%)     | 3 (5.4%)   | 3 (5.4%)   |
|   | <b>Class D (n = 2)</b>  | 1 (50%)             | 0           | 1 (50%)      | 0          | 0          |
| Carbapenemase-producing <i>Klebsiella oxytoca</i> (n = 14)    | <b>Class A (n = 3)</b>  | 2 (66.7%)           | 0           | 0            | 1 (33.3%)  | 0          |
|   | <b>Class B (n = 11)</b> | 4 (36.4%)           | 3 (27.3%)   | 3 (27.3%)    | 1 (9.1%)   | 0          |
|   | <b>Class D (n = 0)</b>  | 0                   | 0           | 0            | 0          | 0          |

HVS = high vaginal swab.

**Table 3**  
Comparison of PCR, CDT, and mCDT for the detection of Class A, B, and D carbapenemase-producing *Klebsiella* spp.

|   |                  | PCR    | CDT        | mCDT       |
|---|------------------|--------|------------|------------|
|   |                  | n = 81 | n = 76 (%) | n = 76 (%) |
| Carbapenemase-producing <i>Klebsiella pneumoniae</i> (n = 67) | Class A (n = 9)  | 9      | 7 (77.8)   | 7 (77.8)   |
|   | Class B (n = 56) | 56     | 54 (96.4)  | 54 (96.4)  |
|   | Class D (n = 2)  | 2      | 2 (100)    | 2 (100)    |
| Carbapenemase-producing <i>Klebsiella oxytoca</i> (n = 14)    | Class A (n = 3)  | 3      | 3 (100)    | 3 (100)    |
|   | Class B (n = 11) | 11     | 10 (90.9)  | 10 (90.9)  |
|   | Class D (n = 0)  | 0      | 0          | 0          |

**Table 4**Performance parameters of mCDT for the differentiation of Class A, B, and D carbapenemase-producing *Klebsiella* spp.

|   |                  | mCDT      |           |           |           |           |           |            |            |
|---|------------------|-----------|-----------|-----------|-----------|-----------|-----------|------------|------------|
|   |                  | TP<br>(n) | TN<br>(n) | FP<br>(n) | FN<br>(n) | SN<br>(%) | SF<br>(%) | PPV<br>(%) | NPV<br>(%) |
| Total carbapenemase-producing <i>Klebsiella</i> spp. (n = 81) | Class A (n = 12) | 10        | 69        | 0         | 2         | 83.3      | 100       | 100        | 97.2       |
|   | Class B (n = 67) | 64        | 14        | 0         | 3         | 95.5      | 100       | 100        | 82.4       |
|   | Class D (n = 2)  | 2         | 79        | 0         | 0         | 100       | 100       | 100        | 100        |
| Carbapenemase-producing <i>Klebsiella pneumoniae</i> (n = 67) | Class A (n = 9)  | 7         | 58        | 0         | 2         | 77.8      | 100       | 100        | 96.7       |
|   | Class B (n = 56) | 54        | 11        | 0         | 2         | 96.4      | 100       | 100        | 84.6       |
|   | Class D (n = 2)  | 2         | 65        | 0         | 0         | 100       | 100       | 100        | 100        |
| Carbapenemase-producing <i>Klebsiella oxytoca</i> (n = 14)    | Class A (n = 3)  | 3         | 11        | 0         | 0         | 100       | 100       | 100        | 100        |
|   | Class B (n = 11) | 10        | 3         | 0         | 1         | 90.9      | 100       | 100        | 75         |
|   | Class D (n = 0)  | 0         | 14        | 0         | 0         | 0         | 100       | 0          | 0          |
| Non-carbapenemase-producing <i>Klebsiella</i> spp. (n = 100)  |                  | 0         | 100       | 0         | 0         | 0         | 100       | 0          | 0          |

TP = true positive; TN = true negative; FP = false positive; FN = false negative; SN = sensitivity; SF = specificity; PPV = positive predictive value; NPV = negative predictive value.

of the patients (87.5%), in their study acquired infection of NDM-1 positive organisms due to their international travel to India.

In the present study, the majority of carbapenemase-producing *Klebsiella* spp. (42%; 34/81) were isolated from sputum samples followed by urine (27.2%; 22/81) and pus (21%; 17/81) samples (Table 2). The results were supported by Radhika and Padmaja (2015) who revealed that 45% (highest) of *Klebsiella* spp. was isolated from sputum samples in their study. In contrast, a study by Mulla et al. (2016) have reported that 45% (highest) of carbapenemase producers in urine samples. Another study by Shawkey et al. (2015) have isolated 54% of carbapenemase-producing strains from blood samples. Majority of carbapenemase-producing *Klebsiella* spp. in this study may be associated with the reason that most of the patients with respiratory tract infections were on mechanical ventilation, which plays a key role to transfer of resistant bacterial strains (Medell and Martinez, 2012).

In this study, a cheaper modification of combined disc test (CDT) (conventional phenotypic method) was developed to differentiate different carbapenemase classes. We observed similar sensitivity (93.8%) and specificity (100%) of both CDT and mCDT (Table 3). However, the cost of consumables (culture media, antibiotic discs, EDTA, PBA) were 4 times cheaper in mCDT because 4 test strains are streaked on a single MHA plate compared to 1 strain in CDT. Furthermore, mCDT is a time- and labor-saving procedure for large number of samples, as required material to be handled is at a cost that is only 25% of CDT.

It has been previously published that sensitivity and specificity of CDT were 94.8% and 100% (Pournaras et al., 2013) and 96.8% and 100% (Athanasios et al., 2010) respectively.

Sensitivity, specificity, positive predictive value, and negative predictive value of mCDT for Class A and Class B carbapenemase-producing *Klebsiella* spp. were found to be 83.3%, 100%, 100%, and 97.2%, and 95.5%, 100%, 100%, and 82.4% respectively. The performance parameters for Class D carbapenemase detection can be changed with substantial number of strains.

In conclusion, mCDT is a simple, labor-saving, and 4 times cheaper method for the differentiation of all 3 carbapenemase classes. Moreover, further evaluation, for other bacterial strains, and implementation of mCDT, especially in resource-limited settings, are recommended.

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## Ethical approval

This study was approved by Institute Ethical Committee (IEC), MMIMSR.

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## Conflicts of interest

Authors declare no conflicts of interest.

## Authors' contributions

Nitin Kumar conceived the presented idea, developed the method, and performed the test. Varsha A. Singh encouraged and supervised the findings of the work. Vikas Beniwal helped in carbapenemase gene detection. All authors contributed to the final manuscript.

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